THE USE OF SPECIFIC ANTIBODY
IN ELECTRON MICROSCOPY

I. Preparation of Mercury-Labeled Antibody

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ABSTRACT

The preparation of antimyosin conjugated with mercury and fluorescein is described. The mercury was introduced to permit visualization of the antibody in the electron microscope. An organic mercurial, tetraacetoxymercuriarsanilic acid, was prepared and coupled to the antibody through the diazonium salt. The fluorescein was coupled through the isocyanate by a modification of the procedure described by Coons and Kaplan. The antibody conjugate retained its specificity of reaction with the tissue antigen. This was demonstrated by the staining pattern obtained in fluorescence microscopy.

INTRODUCTION

The specific reaction of an antibody with its antigen has found histochemical application in the fluorescent antibody technique (1, 2). The fluorescence of the fluorescein-labeled antibody in ultraviolet light reveals the distribution of the specific antigen in a tissue section. Application of this technique to finer problems of cell structure has been limited by the resolution of the light microscope. Extension of the technique to electron microscopy would be possible if the combination of antibody with a specific antigen in cell structures could be visualized in the electron microscope. One means of visualizing antibody in combination with a tissue antigen in the electron microscope is to increase selectively the intensity with which the antibody molecules scatter electrons by introducing heavy metal into the antibody molecules. Such a conjugated antibody may then be visualized as an increase in density, in a particular region of the cell; more ideally, individual conjugated antibody molecules might be resolved. Another means of detecting antibody in combination with a tissue antigen is to observe the change in morphology of a recognizable cell structure due to the adherence of antibody to the antigenic components of the structure. In this latter case no special heavy metal tag is necessary for identification of the antibody since visualization depends on a change in shape rather than an increase in density. This paper will deal with the introduction of heavy metal into the antibody molecule; the following paper will deal with its visualization by electron microscopy. A subsequent paper will deal with the visualization of antibody without specific heavy metal tag.

The following considerations were taken into account in choosing the heavy metal to be introduced into the antibody molecule: (a) The metal must be of as high a molecular weight as possible to be effective in electron scattering. (b) It must be introduced in sufficient quantity to increase visibly the electron opacity of the antibody molecules. (c) It should be covalently linked directly or indirectly to the protein to eliminate the possibility of dissociation from the antibody and subsequent binding to other constituents of the tissue. Non-
specific binding to cell structures through the metal must be eliminated. (d) Introduction of the metal into the antibody molecule must not eliminate the reaction between antibody and antigen. (e) If the metal is first substituted in an organic molecule, a functional group of this molecule must be available for reaction with the antibody. (f) The amount of metal needed to give adequate electron opacity to the final conjugated antibody must not result in insolubility of the modified antibody. (g) The metal must be introduced by means which do not substantially increase the size of the antibody molecule, since this will be one factor determining the precision of localization. (h) The metal should be stable in the electron beam, or methods for stabilizing it must be available. (i) In addition, it would be advantageous to be able to label the antibody with fluorescein as well as the heavy metal to permit direct comparison of results obtained by fluorescence microscopy and electron microscopy.

The method which showed the greatest promise of fulfilling these criteria was mercury. Mercury consequently was coupled to the antibody indirectly through a diazonium link. In addition, fluorescein was coupled to the antibody by use of the fluorescein isocyanate. The resulting modified antibody retained its specificity to its tissue antigens in spite of the extensive coupling.

**METHODS**

For fluorescence microscopy a Reichert Zetopan research microscope was used. Dark-field illumination was obtained using a Zeiss cardioid condenser and a 100 × achromatic oil immersion objective containing a built-in ultraviolet filter. The light source was an Osram HBO-200 lamp. The combination of a 1 mm Corning 5840 and a 1 mm Schott UG-1 filter was used for the incident light. Exposures were of the order of 5 minutes using Kodak type IIa-G spectroscopic plates and Kodak D-19 developer.

**Preparation of Antibody and Normal Globulin**

Myosin was extracted from chicken muscle by the procedure of Mommaerts and Parrish (3) and was further purified by three reprecipitations at an ionic strength of 0.05. Approximately 20 mg of myosin in 4 ml of 0.3 M KCl solution was injected into rabbits intraperitoneally once a week. Serum was collected weekly during the course of injections once the titer was obtained using a Zeiss cardioid condenser and a 100 × achromatic oil immersion objective containing a built-in ultraviolet filter. The light source was an Osram HBO-200 lamp. The combination of a 1 mm Corning 5840 and a 1 mm Schott UG-1 filter was used for the incident light. Exposures were of the order of 5 minutes using Kodak type IIa-G spectroscopic plates and Kodak D-19 developer.

**Preparation of the Organic Mercurial Tetraacetoxymercuriarsanilic Acid**

30 gm of mercuric acetate (0.094 mole) was fused and 3 gm of dry arsanilic acid powder (0.014 mole) was added to the melt. This mixture was kept molten by heating gently for 5 minutes and the viscous melt was then poured into a beaker to cool. These procedures were performed under an efficient exhaust hood because of the highly poisonous mercury and arsanic. The hardened material was then scraped loose and suspended in 100 ml of distilled water. An additional 50 ml of water was added to the straw-colored suspension. 35 ml of 20 per cent NaOH was then added with stirring to give a deep orange-red precipitate. The precipitate was centrifuged out and the resulting pellet was washed twice by resuspension in 90 ml of 3.8 per cent NaOH. After the third centrifugation the pellet was suspended in 100 ml of 20 per cent acetic acid, which converted it to a white flocculent precipitate. This precipitate was centrifuged and the pellet was washed once more with 55 ml of 20 per cent acetic acid by suspension and centrifugation. The final pellet was dried immediately by careful application of vacuum. The dry product was ground to a powder. The rationale of these steps was as follows: The initial sodium hydroxide washes removed any unreacted arsanilic acid by dissolving it as the sodium salt. In addition, any unreacted mercuric acetate was converted to the insoluble mercuric oxide, giving the deep orange-red color. The acetic acid wash then removed the mercuric oxide by converting it to the soluble acetate, leaving only the relatively insoluble reaction product behind.

**Analysis of Product**

The colorimetric method of Cholak and Hubbard (5) was used for mercury analysis. The Schwarzkopf Microanalytical Laboratory, New York, performed C, H, N, and As analyses.

Tetraacetoxymercuriarsanilic acid,

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(NH_2)C_6(HgC_2H_4O_4)AsO_3H_6
\]

theoretically contains: C, 13.4 per cent; H, 1.3 per cent; N, 1.1 per cent; As, 6.0 per cent; Hg, 64.1 per cent. The compound obtained by the synthesis described above contained: C, 12.3 per cent; H, 1.1 per cent; N, 1.0 per cent; As, 5.4 per cent; Hg, 63.7 per cent. This analysis satisfactorily confirms the structure of the synthesized product. The compound darkened at 229–230°C and did not melt at 265°C. If it was left in the bright sunlight decomposition.
occurred and droplets of mercury were formed. It was therefore stored in the dark.

**Preparation of Antibody and Normal Globulin Conjugates**

Preparation of the mercury conjugates required two steps: first, diazotization of the amino group of tetraacetoxymercuiriarsanilic acid to form the diazonium salt; then coupling of this to the antibody. (1) The diazotization reaction was complicated by the low solubility of the mercurial. The procedure finally used was as follows: To 15 mg of the tetraacetoxymercuiriarsanilic acid was added 3.7 ml of 20 per cent acetic acid and a few drops of an NaNO₂ solution (2.73 gm + 100 ml H₂O) to give a slight excess of NaNO₂. The excess NaNO₂ was detected by the appearance of free iodine when a drop of the reaction mixture was mixed with a drop of a solution of KI on a white plate. The reaction mixture was stirred 5 minutes with a magnetic stirrer at 0°C. The mercury compound did not go into solution completely. The solution, while being stirred, gradually became straw-colored and then a rose-purple color. The insoluble material remaining after stirring became intensely colored. (2) The diazonium salt formed was coupled to the antibody as follows: The solution and undissolved material obtained above were added rapidly to 4.0 ml of a solution of the protein (in m/60 PO₄ buffer pH 7.4, 0.1 M KCl) with vigorous stirring at 0°C. Immediately 8.4 ml of cold 6 per cent NaOH solution was added with rapid stirring to bring the pH to 9-10. This mixture was stirred on a magnetic stirrer at 0°C for 1 hour. The soluble material was then dialyzed for 2 days at 10°C against 100 volumes of m/60 PO₄ buffer pH 7.4, 0.3 M KCl, which was changed twice daily.

The resulting globulin-mercury conjugates were further conjugated with fluorescein. The procedure of Coons and Kaplan (6) for the conjugation of fluorescein isocyanate to protein resulted in complete loss of mercury. Therefore the following modified procedure was used: A solution of the globulin-mercury conjugate was brought to pH 9-10 by drop-wise addition of 6 per cent NaOH solution at 0°C, with constant stirring. For each 50 mg of protein, 1 ml of fluorescein isocyanate in acetone solution (Sylvania Chemical Co., Orange, New Jersey) was added slowly, with stirring at 0°C. The pH of the solution was readjusted to 9-10 after addition of the isocyanate. The reaction mixture was stirred for 1 hour on a magnetic stirrer and the pH was readjusted to 9-10 if necessary. It was then stirred overnight and was finally dialyzed at 10°C against m/60 PO₄ buffer pH 7.4, 0.3 M KCl, until no fluorescence was detectable in the dialysate. Finally, the conjugated globulins were dialyzed for 2 days at 10°C against several changes of 25 per cent glycerol in m/60 PO₄ buffer pH 7.4. Analyses showed that no mercury was lost on storage or extensive dialysis. The conjugates were stored at −24°C.

**RESULTS AND DISCUSSION**

The reactions of mercury with organic compounds have been studied extensively but are not clearly understood. Mercury is known to form derivatives with many aromatic organic molecules, substituting directly onto the benzene ring. The preparation of heavily substituted mercury derivatives of aniline and acetanilide has been reported (7). These compounds are extremely insoluble. The procedure described for preparation of the tetraacetoxymercuiriarsanilic acid is similar to that reported for the preparation of the mercury derivative of acetanilide. The purpose of the arsonic acid group was to increase the solubility of the final compound. The presence of the amino group permitted diazotization and coupling of the mercurial to the globulins through the diazonium salt. It was found that the coupling reaction required a pH of 9-10. At this pH, coupling occurs primarily at the tyrosine and tryptophane residues (8, 9) of the protein. The maximum coupling obtained in this way was 32 mercury atoms per molecule of γ-globulin. Subsequent coupling of fluorescein isocyanate to the mercurated γ-globulin by means of the modified procedure already described resulted in loss of some of the mercury, as is shown by the examples in Table I. The variation in the degree of coupling is not now understood.

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**TABLE I**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Protein</th>
<th>Hg atoms per molecule γ-globulin</th>
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<tbody>
<tr>
<td>X-1</td>
<td>Rabbit normal γ-globulin</td>
<td>32.4</td>
</tr>
<tr>
<td>X-2</td>
<td>Rabbit antibody</td>
<td>23.0</td>
</tr>
<tr>
<td>X-1-A</td>
<td>Rabbit normal γ-globulin</td>
<td>19.5</td>
</tr>
<tr>
<td>X-2-A</td>
<td>Rabbit antibody</td>
<td>13.2</td>
</tr>
</tbody>
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The specificity of the staining reaction of the doubly conjugated antibody. × 2300.

a. Normal untreated fibril in phase contrast microscopy.
b. Same fibril as in 1a after treatment with doubly conjugated normal γ-globulin.
c. Same fibril as in 1b in fluorescence microscopy.
d. Same fibril as in 1c after treatment with doubly conjugated antimyosin.
e. Same fibril as in 1d in phase contrast microscopy.
The specific antibody used in this work was that prepared against the muscle protein myosin. The distribution of myosin in the glycerinated myofibril has been studied previously by fluorescence microscopy (10–12). In spite of extensive coupling, the doubly labeled antibody remained specific in its reaction with the antigen.

This was demonstrated by staining a homogenized fibril preparation of glycerinated chicken neck muscle. The homogenization was done in 25 per cent glycerol, M/60 PO₄ buffer pH 7.4, and it was stained with antibody in the same solvent. Staining was done under the microscope by drawing the solutions under the coverslip with filter paper. Photomicrographs were taken at each step in the procedure by phase contrast and fluorescence microscopy. No visible change occurred in the fibril as observed by phase contrast microscopy after treatment with conjugated normal globulin and wash solution, as is shown in Figs. 1a and b. Fluorescence microscopy after treatment with normal globulin also showed no visible nonspecific staining (Fig. 1c). The faint image visible in Fig. 1c is the dark-field image due to a combination of some unfiltered visible light coming through the fluorescence optical system and some autofluorescence of the proteins of the fibril. This photograph was purposely overexposed to bring out any faint staining which might have occurred. This same fibril was then treated with doubly conjugated antibody globulin followed by wash solution. The fluorescence micrograph of this fibril, Fig. 1d, revealed intense alternating bright and dark areas corresponding to fluorescent A bands and non-fluorescent I bands respectively. This is the same pattern of staining obtained with antibody labeled with fluorescein alone (10–12). The corresponding phase micrograph is seen in Fig. 1e. Therefore, the specificity of the staining reaction of the antibody after double conjugation, with both the organic mercurial and the fluorescent labels, has been established.

Another method for labeling serum globulin, to permit electron microscopy of antibody staining, has been described recently by Singer (13). In Singer’s method, the iron-containing protein, ferritin, was coupled to the globulin molecule through a ureide link. The use of ferritin conjugates for the staining of tissues has not yet been reported, but it is obvious that a comparison of the method with that reported in this series of studies would be desirable. One may anticipate two major problems in the use of such ferritin conjugates for studies on cells or tissues: First, the large size of the ferritin molecule limits the precision of localization available with the ferritin-antibody conjugate. Rabbit antibody molecules have been shown, by shadow casting, to be rods with dimensions of approximately 40 by 250 Å (14).

The ferritin molecule itself is a sphere with a diameter of approximately 100 Å (15). In the case of the conjugate labeled with organic mercurial and fluorescein, the size of the conjugate molecule should not be appreciably increased by addition of these smaller molecules. Secondly, because of the large size of the ferritin-antibody conjugate, adequate penetration of the antibody into the tissue will be difficult. This has been a problem even with unconjugated antibody, as is pointed out in paper III of this series.

BIBLIOGRAPHY


